An Innovative Root Inoculation Method to Study *Ralstonia solanacearum* Pathogenicity in Tomato Seedlings

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Accepted for publication 13 November 2017.

ABSTRACT

In this study, we report *Ralstonia solanacearum* pathogenicity in the early stages of tomato seedlings by an innovative root inoculation method. Pathogenicity assays were performed under gnotobiotic conditions in microtube tubes by employing only 6- to 7-day-old tomato seedlings for root inoculation. Tomato seedlings inoculated by this method exhibited the wilted symptom within 48 h and the virulence assay can be completed in 2 weeks. Colonization of the wilted seedlings by *R. solanacearum* was confirmed by using *gus* staining as well as fluorescence microscopy. Using this method,

mutants in different virulence genes such as *hrpB*, *phcA*, and *pilT* could be clearly distinguished from wild-type *R. solanacearum*. The method described here is economic in terms of space, labor, and cost as well as the required quantity of bacterial inoculum. Thus, the newly developed assay is an easy and useful approach for investigating virulence functions of the pathogen at the seedling stage of hosts, and infection under these conditions appears to require pathogenicity mechanisms used by the pathogen for infection of adult plants.

Ralstonia solanacearum is a gram-negative plant-pathogenic bacterium that causes a lethal wilt disease in more than 450 plant species belonging to several monocotyledonous as well as dicotyledonous plant families (Elphinstone 2005; Genin 2010; Hayward 1991; Wicker et al. 2007). The pathogen exceptionally has a wide host range (Coutinho et al. 2000; Jiang et al. 2016; Ozaki and Watabe 2009; Weibel et al. 2016). Several strains of the bacterium have been documented from different parts of the globe, supporting adaptability of the pathogen to wide geographical ranges. Prevalence of noticeable genetic diversity across different strains of the pathogen has led to coining the term “species-complex” in regard to this pathogenic bacterium (Fegan and Prior 2005). Owing to the lethality it causes to its numerous hosts, it was ranked the second most devastating bacterial phytopathogen in the world (Mansfield et al. 2012).

R. solanacearum dwells in soil. In the presence of a host plant, the bacterium attaches to its root, enters the plant, grows, and colonizes inside the xylem, subsequently resulting in the wilting and killing of the plant (Genin 2010). Several important regulatory networks as well as involvement of different protein secretion systems (such as type II and type III) essential for virulence functions in this bacterium have been uncovered (Coll and Valls 2013; Genin and Boucher

2002; Monteiro et al. 2012a; Vasse et al. 2000). In planta gene expression studies of *R. solanacearum* (Ferreira et al. 2017; Jacobs et al. 2012; Puigvert et al. 2017) have provided several clues regarding the adaptive responses of the bacterium within its host. Recently, the roles of diffusible quorum-sensing molecules and extracellular DNases as well as biofilm formations were reckoned to be important for its pathogenic interaction with host (Hikichi et al. 2017; Kai et al. 2015; Mori et al. 2016; Tran et al. 2016). Despite a wealth of exciting findings in regard to *R. solanacearum* biology (Guidot et al. 2014; Hikichi et al. 2017; Marchetti et al. 2010; Peyraud et al. 2016; Remigi et al. 2014), knowledge pertaining to mechanism of its entry into a host, in planta growth and multiplication, and the factors that trigger bacterium’s pathogenicity functions within the host are incomplete. It is also unknown whether the pathogen discriminates between seedling stages and adult stages of its host in natural conditions.

R. solanacearum is considered an attractive model for investigating fundamental aspects of the plant–pathogenic bacteria interaction as well as pathogen–host adaptations (Coll and Valls 2013; Ferreira et al. 2017; Genin 2010; Genin and Boucher 2002). Grown tomato plants are generally utilized as suitable model hosts, where the pathogen is inoculated either by soil drenching or by stem inoculation. In addition, *Arabidopsis thaliana*, *Phaseolus vulgaris* (a distant host plant), and *Mimosa pudica* (a nonhost plant) are other model plants which have been utilized for understanding virulence, host resistance, and evolution of *R. solanacearum* (Deslandes et al. 2002; Guidot et al. 2014; Marchetti et al. 2010; Yang and Ho 1998;). Host plants raised in soil come in contact with soilborne microbes and these associations, in turn, could modulate their fitness (Feau and Hamelin 2017). Therefore, a resident microbial population can affect inferences of *R. solanacearum* virulence assays conducted on such hosts. Furthermore, growing and maintaining large numbers of adult plants requires ample amounts of space and time as well as economic investments. In certain cases, soil drenching and stem inoculation methods were also not found appropriate for analyzing minute virulence differences in a few mutants of *R. solanacearum* (Macho et al. 2010).

Seedling stages of tomato plants have been used for studying *R. solanacearum* pathogenicity on a number of occasions (Artal

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Funding: N. Singh thanks the Department of Biotechnology (DBT), Government of India (GoI) for the fellowship (DBT-JRF/SRF). A. Barman and P. L. Sharma thank the DBT and UExcel grant for post doc and senior research fellowships, respectively. T. Phukan thanks the University Grants Commission, GoI, for the BSR fellowship. K. Kabyashree thanks UGC for the NET-JRF fellowship. S. K. Ray, K. Kabyashree, and R. V. Sonti thank DBT, GoI for the research grant under the twinning project (BT/301/NE/TBP/2012). S. K. Ray, A. Barman, and S. Genin thank CEFIPRA for the Indo-French project grant (4800-B1). S. K. Ray’s lab research is also supported by various departmental projects such as UGC-SAP (DSR II), DST-FIST, and DBT-Strengthening NE.

*The e-Xtra logo stands for “electronic extra” and indicates that eight supplementary figures and eight supplementary tables are published online.

et al. 2012; Kumar 2014; Kumar et al. 2017; Monteiro et al. 2012a; Park et al. 2007; Pradhanang et al. 2000). In fact, there is an interesting recent report on *R. solanacearum* root infection in early stages of *A. thaliana* under gnotobiotic condition (Lu et al. 2018). A gnotobiotic condition for *R. solanacearum* inoculation into tomato seedlings by leaf clipping was recently documented from the author's laboratory (Kumar et al. 2017). Because *R. solanacearum* naturally infects its plant host via the root, its behavior inside the plant may distinctly differ when the pathogen is introduced through other means such as leaf clipping. Therefore, devising an efficient root inoculation method devoid of such constraints was imperative. In this context, here, we report an innovative method of *R. solanacearum* root inoculation into 6- to 7-day-old tomato seedlings which is equally efficient in causing disease in different cultivars of tomato. The method has been successfully implemented in studying pathogenicity functions of *R. solanacearum* by recruiting mutant strains for important virulence regulators such as *hrpB* and *phcA* in tomato seedlings. The method described here is reasonably rapid, easy, and cost effective as well as requiring less inoculum of bacteria. Considering these attributes, this method of inoculation is anticipated to help significantly in understanding intricate molecular mechanisms of *R. solanacearum* virulence in seedling stages of hosts in the near future.

MATERIALS AND METHODS

Bacterial strains, growth media, and culture conditions.

Bacterial strains used in the entire work have been listed in Table 1. Growth medium used for the wild-type *R. solanacearum* F1C1 (Kumar et al. 2013) and derivative mutant strains as well as *Pseudomonas putida* was BG (Bacto agar-glucose) medium (Boucher et al. 1985) supplemented with 0.5% glucose. Incubation temperature for *R. solanacearum* strains and *P. putida* was 28°C. *Escherichia coli* and *Bacillus subtilis* strains were grown in Luria-Bertani (LB) medium (Bertani 1952) at 37°C; 1.5% agar was added in case of solid medium as and when necessary. Concentrations of different antibiotics used were as follows: spectinomycin (50 µg ml⁻¹), ampicillin (50 µg ml⁻¹), rifampicin (50 µg ml⁻¹), and gentamycin (50 µg ml⁻¹). All media components and antibiotics used in this work were bought from Himedia.

Germination of tomato seedlings for inoculation. Tomato seed of different cultivars recruited in this study were presoaked in sterile distilled water for 2 days. This was followed by spreading the seed on sterile wet tissue paper in a plastic tray and allowing them to germinate in a growth chamber (Orbitek) maintained at 28°C, 75% relative humidity (RH), and a 12-h photoperiod. Sterile distilled water was sprinkled regularly to sustain the germination process for 6 to 7 days. Age of the seedling was defined from the day the seed were kept for germination on the wet tissue paper bed.

Preparation of bacterial inoculum. *R. solanacearum* F1C1 was streaked on a BG agar plate. Freshly grown *R. solanacearum* (F1C1) colonies were added to 50 ml of BG broth with a sterile loop and allowed to grow in a shaking incubator (Orbitek) maintained at 28°C and 150 rpm. After 24 h, bacterial cultures were centrifuged at 4,000 rpm (3,155 × g) for 15 min at 4°C. Bacterial pellets were resuspended in an equal volume of sterile distilled water to obtain a concentration of approximately 10⁹ CFU ml⁻¹. *P. putida* was grown in BG broth, similar to *R. solanacearum*, at 28°C, whereas LB broth was used for culture of *E. coli* and *B. subtilis* at 37°C in a shaking incubator maintained at 150 rpm. All of the inoculums were prepared following the same procedure used for *R. solanacearum* F1C1 mentioned above.

Root inoculation of *R. solanacearum* in tomato seedlings.

Approximately 15 to 20 ml of *R. solanacearum* F1C1 inoculum (approximately 10⁹ CFU ml⁻¹) was taken in a sterile container (Fig. 1A and B). From the germinated seedling tray, 6- to 7-day-old tomato seedlings were picked one at a time. Roots of each seedling were then dipped in the bacterial inoculum (up to the root-shoot junction) followed by transfer of the seedling to an empty 1.5- or 2.0-ml sterile microfuge tube. All of the seedlings were inoculated by the same procedure. The root-dip-inoculated seedlings transferred to microfuge tubes were subjected to air exposure for approximately 5 min prior to addition of 1 to 1.5 ml of sterile water to each tube. Exposure of the inoculated roots to air at this step was found to be critical for *R. solanacearum* pathogenicity in tomato seedlings.

In all of the experiments, 40 seedlings at minimum were taken for each bacterial inoculum used. In the control set, 40 seedlings were mock inoculated with sterile distilled water following the same

TABLE 1. Bacterial strains and plasmids used in this study

Sl number	Strain	Characteristics ^a	Reference or source
<i>Ralstonia solanacearum</i>			
1	F1C1	Wild-type virulent <i>R. solanacearum</i> strain (phylotype I) isolated from wilted chili plant collected from a nearby field of Tezpur University, Tezpur, India	Kumar et al. 2013
2	TRS1001	<i>rif^r</i> ; <i>Rif^r</i> ; <i>Vir⁺</i> strain derived from F1C1; the strain was selected as a spontaneous <i>Rif</i> mutant from F1C1 culture	Kumar 2014
3	TRS1002	<i>rif^r</i> <i>zxx::Tn5gusA11</i> ; <i>Gus⁺</i> , <i>Rif^r</i> , <i>Spc^r</i> , <i>Vir⁺</i> ; this strain is derived from TRS1001 after <i>Tn5gusA11</i> insertion in an unknown locus in the genome	Kumar 2014
4	TRS1012	<i>hrpB::Ω</i> ; <i>Spc^r</i> , <i>HrpB</i> deficient, <i>Vir⁻</i> , hypersensitive response deficient, derived from F1C1	This work
5	TRS1013	<i>phcA::Ω</i> ; <i>Spc^r</i> , <i>PhcA</i> deficient, exopolysaccharide deficient, hypermotile, derived from F1C1	This work
6	TRS1014	<i>pilT::pNST001</i> ; <i>Amp^r</i> and <i>Gen^r</i> , <i>PilT</i> deficient, <i>Vir⁻</i> , twitching motility deficient, derived from F1C1	This work
7	TRS1015	<i>rpoN2::pNSN2001</i> ; <i>Amp^r</i> and <i>Gen^r</i> <i>RpoN2</i> deficient, <i>Vir⁺</i> , derived from F1C1	This work
8	TRS1016	<i>Gen^r</i> , mCherry-tagged F1C1	This work
<i>Escherichia coli</i> and other bacteria			
1	DH5α	F ⁻ Φ80 <i>lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK⁻, mK⁺)</i>	Lab collection
2	N4T	<i>Pseudomonas putida</i>	Lab collection
3	C6a	<i>Bacillus subtilis</i>	Lab collection
Plasmids			
1	pGEMT	<i>Amp^r</i> ; cloning vector	Promega
2	pTZ57R/T	<i>Amp^r</i> ; cloning vector	Thermo Scientific
3	pCZ367	<i>Amp^r</i> ; <i>Gen^r</i> ; insertional vector with <i>lacZ</i> reporter	Cunnac et al. 2004
4	pNST1	<i>pTZ57R/T::pilT</i>	This work
5	pNSN2	<i>pTZ57R/T::rpoN2</i>	This work
6	pNSN2001	<i>pCZ367::rpoN2</i>	This work
7	pNST001	<i>pCZ367::pilT</i>	This work

^a *Rif^r*, *Spc^r*, *Amp^r*, and *Gen^r* indicate resistant to rifampicin, spectinomycin, ampicillin, and gentamycin, respectively. *Vir⁺* and *Vir⁻* indicate virulence proficiency and virulence deficiency, respectively.

steps as mentioned above. All of the inoculated seedlings along with controls were transferred to a growth chamber maintained at 28°C and 75% RH, with a 12-h photoperiod. Seedlings were analyzed for disease progression from the next day onward till the seventh day postinoculation and findings were recorded.

Seedlings of four commercially available tomato cultivars—namely, Akhilesh (Param Hybrid Seed), Vijay (Param Hybrid Seeds), Durga (selection -22), and Durga (Ruby)—were tested for *R. solanacearum* pathogenicity by the root inoculation method, as described above.

Root inoculation of nonpathogenic bacteria such as *B. subtilis*, *P. putida*, and *E. coli* in tomato seedlings was also done as above.

Root inoculation of tomato seedlings with different titers of *R. solanacearum* F1C1. Seven-day-old tomato seedlings of Durga (selection -22) were inoculated with different dilutions (10^9 to 10^6) (i.e., 10^9 , 10^8 , 10^7 , 10^6 , 10^5 , 10^4 , and 10^3 CFU ml⁻¹ inoculum) of *R. solanacearum* F1C1 by the root inoculation technique described above to determine the effect of different titers of pathogen on disease progression. Sets of 40 seedlings were recruited in

Fig. 1. A, Pictures describing different steps involved the root inoculation method to study *Ralstonia solanacearum* pathogenicity in tomato seedlings. **(a)** Germination of tomato seed on a sterile and wet tissue paper bed. Prior to spreading, seeds were washed and soaked for 24 h. Seeds were allowed to germinate in a growth chamber at 28°C and 75% relative humidity up to the seedling stage. **(b)** Germinated seedlings in a plate after 6 to 7 days. **(c)** A 7-day-old tomato seedling is taken out. **(d)** Root inoculation of tomato seedling in pathogen inoculum by dipping the root. **(e)** Each inoculated seedling transferred to sterile empty microfuge 1.5- to 2-ml tubes. After approximately 5 min of exposure to air, 1.0 to 1.5 ml of sterile water was added to the microfuge tube. The tray containing the microfuge tubes having the seedlings was kept inside the growth chamber. **(f)** Within 48 h, infected seedlings started wilting. **(g)** After 7 days, 80 to 90% of infected tomato seedlings were wilted or had died (lower side) in comparison with water control (upper side). **B,** Schematic representation of the *R. solanacearum* pathogenicity in tomato seedlings by the root inoculation method. The 6- to 7-day-old tomato seedlings were root dipped in bacterial suspension and then transferred to microfuge tubes. After approximately 5 min of exposure to air, water was added to the microfuge tube and the tube incubated in the growth chamber. Wilting symptoms started appearing from the second day postinoculation onward. To observe maximum wilting in the seedlings, sequence number 2 is to be followed. In sequences 1 and 3, infection was found to be inconsistent and low.

each dilution inoculation. Bacterial pelleting was performed as stated in previous sections. The resuspended bacterial pellets were serially diluted to obtain *R. solanacearum* (F1C1) at approximately 10^9 to 10^3 CFU ml⁻¹ of by adding sterile distilled water prior to inoculation. Seedlings were analyzed for disease progression till the seventh day postinoculation and data were recorded.

Transformation in *R. solanacearum* F1C1 strain. The protocol used to transform F1C1 followed the method described by Gonzalez et al. (2011), with modifications in glycerol concentration used. F1C1 was grown in minimal medium containing 10% glycerol as a sole carbon source for 48 h at 28°C. The bacterial inoculum (50 µl) was mixed with 1 to 2 µg of linearized genomic DNA as well as plasmid DNA and spotted on 0.45-µm sterile nitrocellulose membrane kept over a BG agar plate without glucose. After 48 h of incubation at 28°C, bacteria were scooped out, resuspended in 150 µl of distilled water, and plated on the selection medium containing appropriate antibiotics.

Creation of mCherry-tagged *R. solanacearum* F1C1 and its colonization study in tomato seedlings. The plasmid pRCG_Pps-mcherry carrying a constitutive mCherry gene expression reporter (Capela et al. 2017; Monteiro et al. 2012b) was linearized with restriction enzyme *ApaI* and naturally transformed into *R. solanacearum* F1C1. mCherry-labeled transformants were selected on BG agar plates containing gentamycin antibiotic. One of the mCherry-labeled strains (TRS1016) was cultured in BG broth containing an appropriate antibiotic. TRS1016 culture was pelleted down and inoculum of the former at 10^9 CFU/ml was prepared by the same method described for *R. solanacearum* above. TRS1016 inoculum was used for root inoculation of 7-day-old tomato seedlings, as stated above. After 3 days postinoculation, tomato seedlings were surface sterilized following the method of Kumar et al. (2017). Sterilized seedlings were observed under the fluorescence microscopy (EVOS FL; Life Technologies) at ×40 magnification adjusted in a red fluorescent protein filter.

Creation of *pilT* and *rpoN2* mutants of *R. solanacearum* F1C1. Taking the reference genome of GMI1000 from the LIPM database (<https://iant.toulouse.inra.fr/>), primers were designed for partial amplification of *rpoN2* and *pilT* gene homologs in the *R. solanacearum* F1C1 strain. In all of the sets of primers designed, forward primers contained a *HindIII* restriction site and the reverse primers were incorporated with an *XbaI* restriction site at their 5' ends. Primers sequences are given in Supplementary Table S8. Primers (5'-GCCAAGCTTGCTGCCAAGAACAAGCGTCT-3' and 5'-GCCTCTAGATCCCGCAGCGCCGATT-3') were used for amplification of an approximately 500-bp amplicon of the *pilT* gene homolog in F1C1. This amplicon was sequenced for confirming homology with the *pilT* as well as *rpoN2* sequences of the GMI1000 strain. The amplicon was ligated to a T-A cloning vector pTZ57R/T (Thermo Fisher Scientific) to get a construct pNST1 (pTZ57R/T::*pilT*_{F1C1}) following the manufacturing company's instructions. pNST1 was subjected to restriction digestion with *HindIII* and *XbaI* enzymes simultaneously. The resulting approximately 500-bp amplicon from the previous step was gel extracted and, subsequently, ligated to the pCZ367 (Cunnac et al. 2004) vector that harbors a promoterless *lacZ* reporter gene and ampicillin and gentamycin selection markers. Prior to ligation setup, pCZ367 vector was linearized with the same pair of restriction enzymes used in digestion of the amplicon. Recombinant plasmid pNST001 (pCZ367::*pilT*_{F1C1}) was isolated from transformed DH5α cells followed by confirmation of the cloning step with digestion of pNST001 with *HindIII* and *XbaI* enzymes.

Primers (5'-GCCAAGCTTGCGACCGAATTTGCACAGG-3' and 5'-GCCTCTAGACGTCTTCGGCCTCGATCAT-3') were used to amplify an approximately 1.3-kb amplicon of *rpoN2* homolog in F1C1. This amplicon was confirmed for homology with the *rpoN2* gene in GMI1000 by nucleotide sequencing. This amplicon was first cloned into pTZ57R/T vector to obtain pNSN2 (pTZ57R/T::*rpoN2*_{F1C1}) and, subsequently, the same amplicon was ligated into pCZ367 vector to obtain vector construct pNSN2001 (pCZ367::*rpoN2*_{F1C1}). Cloning steps were the same as discussed above.

The recombinant vector constructs pNST001 and pNSN2001 were naturally transformed into *R. solanacearum* F1C1 strain to create insertion mutations in *pilT* and *rpoN2* gene homologs of F1C1, respectively. Successful transformants were selected on BG agar plates containing ampicillin and gentamycin antibiotics. Transformants were checked for positive 5-bromo-4-chloro-3-indolyl-β-D-galactoside activity. Insertion mutations in *pilT* and *rpoN2* genes of successful transformants were confirmed by polymerase chain reaction. The phenotype of the *pilT* mutant was also studied for twitching motility deficiency (Supplementary Fig. S7), following the method of Liu et al. (2001). One insertion mutant from each of the above—namely, TRS1014 (F1C1::pCZ367::*pilT*) and TRS1008 (F1C1::pCZ367::*rpoN2*)—was recruited for subsequent experiments.

Creation of *hrpB* and *phcA* mutants of *R. solanacearum* F1C1. To create *hrpB* and *phcA* mutations in the F1C1 background, genomic DNA samples from the *hrpB* mutant (GMI1525) (Genin et al. 1992) and *phcA* mutant (GMI1605) (Genin et al. 2005), both created in the GMI1000 strain background, were used to naturally transform wild-type F1C1 individually. Transformants for both types were selected on BG agar plates supplemented with spectinomycin antibiotic. Two of the transformants from a previous step—TRS1012 (*hrpB* mutant F1C1) and TRS1013 (*phcA* mutant F1C1)—were recruited in subsequent studies. *hrpB* mutants of F1C1 were found to be deficient to elicit the hypersensitive response in tobacco leaves after infiltration, as well as virulence deficient in tomato seedlings by root inoculation, unlike the wild-type F1C1 (Fig. 2). The *phcA* mutant colonies were transparent due to being deficient for exopolysaccharide production, exhibited very high motility on semisolid agar medium, and were virulence deficient, unlike the wild-type F1C1 (Fig. 2).

RESULTS

***R. solanacearum* pathogenicity in tomato seedlings by a root inoculation method.** An initial observation in the author's laboratory regarding development of pathogenicity symptoms in 6- to 7-day-old tomato seedlings under gnotobiotic root inoculation of *R. solanacearum* confirmed the susceptibility of early stages of tomato seedlings toward the pathogen (Kumar 2014). In this setup, each tomato seedling (S22 Evergreen variety) kept in a 1.5-ml microfuge tube containing 1.0 ml water was directly inoculated with *R. solanacearum* suspension. Subsequently, this root inoculation method was found to be not effective against seedlings of other tomato cultivars tried in the laboratory. However, the seedlings of different tomato cultivars were susceptible to *R. solanacearum* F1C1 infection by the leaf-clipping inoculation method developed in the authors' laboratory (Kumar et al. 2017). Therefore, devising an efficient root inoculation method devoid of such constraints was imperative.

Fig. 2. Virulence data of F1C1, *hrpB*, *phcA*, and *rpoN2* strains in the root inoculated tomato seedlings. The x-axis represents the days postinoculation and y-axis represents the percentage of wilted seedlings. It is distinctly clear that the *hrpB* mutant is nonpathogenic whereas the *phcA* mutant is significantly reduced for virulence, and the *rpoN2* strain is like the F1C1 wild type.

Although our efforts to find an effective root inoculation method in 6- to 7-day-old seedlings were continuing, in a serendipitous way we observed that a subtle change in inoculation approach significantly influenced *R. solanacearum* pathogenicity in tomato seedlings. *R. solanacearum* inoculated by the devised root inoculation method was proficient in causing pathogenicity in young tomato seedlings (Fig. 1A). Two steps in the inoculation process were found to be crucial (Fig. 1B). First, immersion of the tomato seedling root in *R. solanacearum* suspension followed by exposure of the root to air had tremendous influence on the pathogenicity symptoms developed in the seedlings. In our experiments, the air exposure time has been kept at 5 min, although an instant air exposure was also sufficient to result in virulence of the tomato seedlings. Second, direct addition of *R. solanacearum* suspension to roots of tomato seedlings already submerged in water resulted in lesser or inconsistent disease symptoms. Another method (i.e., directly transferring the seedlings into a microfuge tube already containing *R. solanacearum* suspension) also did not result in very high numbers of wilting symptoms of the seedlings. Disease progression as well as pathogenicity due to *R. solanacearum* were investigated in tomato seedlings after the root inoculation. It was notable to observe that the wilting symptoms as well as death of some inoculated seedlings appeared within 35 to 48 h postinoculation. In fact, observation of pathogenicity symptoms by the root inoculation method is faster than the leaf clipping inoculation method, in which the disease symptoms appeared on the third day postinoculation (Kumar et al. 2017). On the seventh day postinoculation via this root inoculation method, approximately 80 to 90% of the inoculated seedlings were found to be dead. To confirm that the death of the inoculated seedlings by this method was specific to *R. solanacearum* inoculation, seedlings were also inoculated with few nonpathogenic bacteria such as *P. putida*, *B. subtilis*, and *E. coli*. After the seventh day postinoculation, none of the seedlings inoculated with these nonpathogenic bacteria exhibited disease symptom (Supplementary Fig. S1; Supplementary Table S1). This outcome indicated that death of the seedlings occurred due to *R. solanacearum*

F1C1 via this mode of inoculation. Further *R. solanacearum* F1C1 pathogenicity of similar magnitude could be observed in three other tomato cultivars: Durga (Ruby), Akhilesh, and Vijay (Supplementary Fig. S2; Supplementary Table S2). We further studied F1C1 pathogenicity in tomato seedlings by root inoculation as a function of the pathogen concentration in the inoculum. Bacterial concentration varied from approximately 10^9 to 10^3 CFU ml⁻¹. Disease symptoms were observed distinctly up to approximately 10^5 CFU/ml. It is also observed that the pathogenicity and disease progression magnitude decreased as bacterial concentration in the inoculum decreased (Supplementary Fig. S3; Supplementary Table S3).

The association of F1C1 with tomato seedling inoculated by this method was studied further by using *R. solanacearum* F1C1-derived strain TRS1002 (*gus* marked) as well as TRS1016 (mCherry marked) to study bacterial colonization in the seedlings. Bacterial colonization in the infected seedlings was observed from root to the shoot regions (Fig. 3A and B). This suggested that, after the inoculation, the bacterium migrated from root to shoot regions during the infection process and resulted in pathogenesis.

The root inoculation method can be used to study *R. solanacearum* virulence functions. To further evaluate that this root inoculation method is useful in studying virulence functions of *R. solanacearum*, we inoculated tomato seedlings with different *R. solanacearum* mutants such as *hrpB* (TRS1012), *phcA* (TRS1013), and *rpoN2* (TRS1015) (Table 1). As anticipated, the *hrpB* mutant was nonpathogenic, the *phcA* mutants was found to be significantly reduced for virulence, and the *rpoN2* mutant exhibited virulence proficiency (Fig. 2; Supplementary Fig. S4; Supplementary Table S4). The virulence phenotypes of *hrpB*, *phcA*, and *rpoN2* mutants were in concordance with the virulence phenotype data reported earlier in adult tomato plants (Ray et al. 2015). A characteristic yellowish color of the cotyledon leaves was observed in the case of seedlings inoculated with the *phcA* mutant. However, a leaf-clip-inoculated *phcA* mutant had no such manifestations. Future investigation may reveal the reason behind this difference.

It has been reported in the literature that a twitching-motility-deficient strain (*pilT* mutant) of *R. solanacearum* is virulence deficient in tomato plants inoculated by soil drenching (Kang et al. 2002; Liu et al. 2001). We created a *pilT* mutant of F1C1 in this study (TRS1014), which was deficient for twitching motility. TRS1014 was found to be moderately virulence deficient in tomato seedlings, unlike *hrpB* and *phcA* (Fig. 4; Supplementary Fig. S5; Supplementary Table S5). This proved that the root inoculation method in tomato seedlings is useful to discriminate between severely and moderately virulence-deficient strains of *R. solanacearum*. Interestingly, TRS1014 was further found to be virulence proficient by the leaf-clip inoculation method in tomato seedlings (Supplementary Fig. S6; Supplementary Table S6). This might be due to differential requirements of twitching motility during the two modes of infection.

DISCUSSION

In this work, we are documenting a root inoculation method to study *R. solanacearum* pathogenicity in early stages of tomato seedlings under gnotobiotic conditions. This method is effective for performing the pathogenicity assay in seedlings of different tomato cultivars. It was also found useful for studying the known virulence functions of *R. solanacearum* such as *hrpB*, *phcA*, and *pilT*. Through this inoculation process, *R. solanacearum* strains tagged with *gus* and mCherry were recruited for observing bacterial colonization in tomato seedlings which manifested colonization of the bacteria in root as well as shoot regions. The method is reasonably simple, easy, and rapid. Because this involves less inoculum of bacteria as well as less space, large-scale screening of tomato seedlings for an *R. solanacearum* virulence assay is possible by this method. It is pertinent to note that, previously, root inoculation of this pathogen has been performed in plantlets of *Medicago truncatula* as well as tomato by germinating seed under sterile conditions in the presence

Fig. 3. **A**, X-gluc staining of the seedlings root inoculated with *Ralstonia solanacearum* marked with *gus*. In this picture, the staining of seedling 2 suggests that it is *gus* positive. For the *gus* assay, this tomato seedling was root inoculated with a *gus*-marked *Ralstonia solanacearum* strain (TRS1002). The staining in the root and shoot regions confirmed the bacterial colonization in the pathogen-infected seedling while other tomato seedlings (such as seedling 1) were control seedlings, where no bacteria were inoculated; these appeared as *gus* negative. **B**, Tracking the presence of *R. solanacearum* in tomato seedlings inoculated with mCherry-labeled bacteria. For further confirmation of pathogen colonization inside infected tomato seedlings, we inoculated an m-Cherry-tagged *R. solanacearum* strain (TRS1016) in tomato seedlings through the root. In this image, fluorescence was observed in the stem region, confirming the presence and colonization of m-cherry-tagged *R. solanacearum* inside the tomato seedling.

or absence of nutrients (Vailleau et al. 2007; Vasse et al. 1995). Because of the involvement of tissue-culture-based techniques, these methods will not be easy for doing pathogenicity study in large scale. Recently, Lu et al. (2017) reported *R. solanacearum* root infection in early stages of *A. thaliana* under gnotobiotic condition.

The extensive pathogenicity of *R. solanacearum* in 6- to 7-day-old tomato seedlings within 48 h of root inoculation is a demonstration of its virulence in very early stages of plant growth. Though similar pathogenicity in tomato seedlings was reported earlier by the same authors using leaf-clipping inoculation, the pathogenicity in seedlings by root inoculation holds much more significance than the leaf-clipping inoculation because the pathogen enters the host plant through its roots during natural infection. An interesting point in regard to leaf clipping, where disease symptoms in tomato seedlings were noticed to begin at the point of inoculation and then progress downward (Kumar et al. 2017), is that, through this root inoculation process, disease appearance could be observed in the shoot region without any visible sign of effects at the root region. Furthermore, the characteristic bending of the upper shoot region in the beginning of the disease in seedlings was observed by root inoculation but was not observed in seedlings by leaf-clipping inoculation. This indicates that infection in the tomato seedlings by the two modes of inoculation may not be identical. This is supported by virulence deficiency of the *pilT* mutant through the root inoculation method but not by the leaf-clipping inoculation method. A recent report claims that *R. solanacearum* has to overcome the host root-cell-fabricated nucleic acid network in order to successfully invade the root (Tran et al. 2016). It is likely that the *R. solanacearum* entry mechanism through the root might be more complicated than by any shoot inoculation mechanism such as leaf clipping. It is pertinent to note that, previously, *R. solanacearum* mutants deficient in swimming motility or in aerotaxis were reported to be virulence deficient through the method of root inoculation by soil drenching, whereas they were virulence proficient when inoculated by the petiole-cut method in tomato plants (Tans-Kersten et al. 2001, 2004; Yao and Allen 2007).

The difference in the number of tomato seedlings dying as a function of bacterial concentration in the inoculum is interesting. It indicates that the initial population of bacteria during infection and their growth inside the seedlings are important for the disease symptoms. How the pathogen population affects the disease in plants in the case of *R. solanacearum*, which is a systemic pathogen in comparison with other bacterial pathogens that are tissue specific, is an interesting question for future research.

In the method presented here, immediate exposure of roots to air after instant dipping in the bacterial inoculums was found to be crucial for aggressive infection and disease progression in tomato

seedlings. The importance of exposure to air was a serendipitous finding in our study. The air exposure time for roots in our experiment was set for 5 min (Fig. 1A and B), although even an instantaneous exposure to air is sufficient to cause pathogenicity symptoms in tomato seedlings. Although the precise role of air exposure is unknown, claims of Yao and Allen (2007) regarding involvement of aerotaxis in *R. solanacearum*–tomato plant interaction might indicate involvement of an identical mechanism during the pathogen–tomato seedling impingement. The authors (Yao and Allen 2007) tested *R. solanacearum* root colonization by incubating tomato seedlings in bacterial inoculums for 30 min, although there was no mention of virulence in them. A probable role of air exposure or aerotaxis also may be predicted in the root-dip assay conducted by Park et al. (2007) in 6-week-old tomato plants, where they incubated tomato plant roots in bacterial suspension for an equal duration as in the case of Yao and Allen (2007) before transferring the plants to soil. Similarly, in their pathogenicity assay, Maji and Chakrabarty (2014) immersed surgically wounded roots of tomato plantlets in an *R. solanacearum* suspension for 3 h. Unknowingly, the transient period between transfers of the plants from bacterial suspension to the respective culture conditions in their experiments might have played a role during infection progression. In addition to the above, during our infection study, we observed an interesting infection behavior of *R. solanacearum* in the case of prewet tomato seedlings. When tomato seedlings were incubated for 24 h in a microfuge tube with the root region submerged in sterile water, then inoculated with *R. solanacearum* by the root inoculation method, the seedlings were surprisingly found to be less susceptible to wilting symptoms (Supplementary Fig. S8). However, bacterial colonization was observed inside the inoculated prewet tomato seedlings. It is interesting to note that the tomato seedlings under the same prewet conditions were susceptible to *R. solanacearum* pathogenicity when inoculated by the leaf-clipping method. Whether *R. solanacearum* is deficient in its attachment to the roots of the prewet tomato seedlings will require further investigation.

Considering the potential benefits of the root inoculation method described here, we anticipate that this inoculation method will help the world scientific community to address several fundamental questions pertaining to *R. solanacearum* interaction with seedling stages of the host and aid in foreseeing mechanisms of virulence in adult plants. This method might turn out to be instrumental in devising suitable biocontrol measures against the wilt pathogen in the immediate future.

ACKNOWLEDGMENTS

We thank L. Sahoo, IIT-Guwahati, India for the kind gift of the tobacco plant in which the hypersensitive response assay could be performed, and the *Phytopathology* editor and all three reviewers for their kind comments and suggestions on the manuscript.

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Fig. 4. *pilT* is virulence deficient by root inoculation. The x-axis represents the days postinoculation and the y-axis represents the percentage of wilted seedlings. It is distinctly clear that the *pilT* mutant is reduced for virulence by root inoculation in comparison with the F1C1 wild type. We compared the F1C1 wild type with the *pilT* mutant in two different concentrations of bacteria in the inoculum (100-fold dilution and 102-fold dilution). In both concentrations, *pilT* was found to be reduced for virulence.

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